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Protein recovery from yellow peas (*Pisum sativum* L.) for enhanced processing sustainability and functional properties

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This research focuses on sustainable protein recovery methods from a new yellow pea variety by examining alternative pH-shifting processes. The study focuses on reducing water consumption during alkaline extraction by adjusting solid-liquid ratios, and evaluating the impact of various isoelectric precipitants, including lactic acid and lactic acid bacteria (*Lactobacillus plantarum* and *Lactobacillus lactis*), on the functional and antioxidant properties of products across a wide range of pH values. It was here found that the process alternative with three 1:10 (w/v) extraction cycles and lactic acid bacteria as precipitant agent achieved high process productivity (0.36 kg protein product/kg pea flour) and low specific water consumption (94.9 kg water/kg protein product). No significant differences were observed in protein content and yield when compared to other flour-water ratios with higher water consumption or less eco-friendly precipitants. Products precipitated with lactic acid bacteria formed stable emulsions even at the isoelectric point, exhibited superior free radical scavenging activity, although solubility and water holding capacity were lower, and no differences were noted in oil holding capacity, foaming capacity, and foam stability.

Key words: protein extraction, alkaline water, pH, precipitation, lactic acid bacteria

Introduction

Yellow peas, known for their low allergenicity, high nutritional value, and abundance (Gao et al. 2020, Hertzler et al. 2020), are a particularly appealing source of plant-based protein, constituting 20–25 % of their composition (Emkani et al. 2022). This makes them rich in essential amino acids like lysine, leucine, isoleucine, and phenylalanine, contributing to a low-fat content of 1.5–2.0 % (Boye et al. 2010a, Shanthakumar et al. 2022). However, compared to animal proteins, they exhibit a lower biological value due to reduced sulphur-containing amino acids (Lam et al. 2018, Shanthakumar et al. 2022).

The cultivation of peas in Argentina has witnessed a surge due to their ability to fix nitrogen, reducing production costs and greenhouse gas emissions (Vita Larrieu and Prieto 2022, Khazaei et al. 2019). This increase, reaching a historic production record of 225 000 tons, reflects a growing interest in both enhancing the industrialization chain for peas and bolstering the social economy in the sector (Di Yenno et al. 2022). Several studies have explored protein extraction from legumes using the pH-shifting process, involving solubilization at pH 8–11 and subsequent acidification (Klupšaitė and Juodeikienė 2015, Accoroni et al. 2020, Gao et al. 2020, Kolpakova et al. 2021). While effective, this method's drawback lies in its significant water consumption during extraction, posing environmental and economic challenges (Hadnađev et al. 2017, Cheng et al. 2018). Furthermore, the use of a food-grade precipitant agent in the precipitation stage needs careful consideration for implementation of the protein recovery process in the food industry (Emkani et al. 2021, Pei et al. 2022).

In this context, the goal of this study is to evaluate the performance of various alternatives at the pH-shifting process for the production of a protein product from a new variety of yellow peas. This work focuses on the reduction of the amount of water consumed at the extraction stage for obtaining each kilogram of protein product, and the usage of a GRAS (generally recognized as safe) precipitant agent (lactic acid and/or lactic acid bacteria) in the precipitation stage to enhance the functional and antioxidant properties of the resultant protein products.

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Materials and methods

Experimental design

In pursuit of optimizing the pH-shifting process for a new yellow pea variety, this study focused on evaluating the reduction of water consumption during extraction and exploring different precipitant agents' impact on protein recovery. The experimental design comprised 15 runs, which followed the processing steps shown in Figure 1 and further detailed in the following sections. The experimental runs included:

- A preliminary exploration of the alkalinization pH values in the extraction stage, which were set at 8.5 and 10 since higher pH values may improve the protein recovery performance.

- The evaluation of the adoption of different combinations of solid-liquid ratios in each of the 3 cycles of the alkaline extraction stage, as detailed in the Methods' section (Alkaline extraction), and the usage of different precipitant agents (hydrochloric acid, lactic acid, or a combination lactic acid bacteria and lactic acid) in the isoelectric precipitation stage, as detailed in the Methods'section (Isoelectric precipitation).

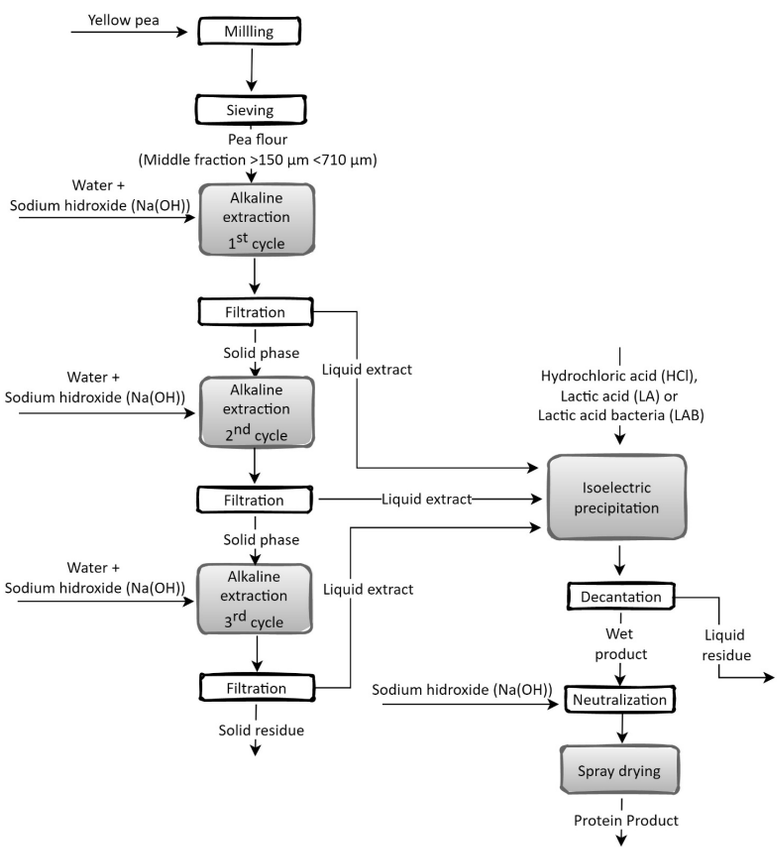

Fig. 1. Experimental methodology for the recovery of proteins from yellow peas, using different extraction conditions and precipitant agents

Materials

The new yellow pea variety (*Pisum sativum L.*) here used were developed by INTA (EEA Oliveros, National Institute of Agricultural Technology, Santa Fe, Argentina) and UNR (Faculty of Agricultural Sciences, Rosario National University, Rosario, Argentina). Samples were stored in sealed bags at freezer temperatures (–18 °C) until processing.

Analytical and food grade chemicals (Ciccarelli, Argentina) and GRAS commercial freeze-dried lactic acid bacteria starters (*Lactobacillus plantarum*, *Lactobacillus lactis*) (CHRHansen, Denmark) were used.

Methods

Pre-processing and characterization of yellow peas

Yellow peas were ground at room temperature using a laboratory mill (Bühler, Germany), and then they were sieved through ASTM-standard sieves to achieve a particle size that passed through a 25-mesh (710 µm) sieve and was retained by a 100-mesh (150 µm) sieve.

The nitrogen content of the yellow peas was assessed through AOAC method 2001.11 (AOAC 2005), where the total protein content was derived by multiplying the total nitrogen by 6.25 and reported as % db (i.e. on a dry basis or moisture-free basis). The protein solubility index in KOH (potassium hydroxide) of the yellow peas was determined following the procedure described by Araba and Dale (1990). The moisture content of the yellow peas was determined using AOAC method 925.10 (AOAC 2005) and reported as % wb (i.e. on a wet basis).

Alkaline extraction

The extraction was performed in a batch extractor with continuous stirring (Precylec, Argentina) consisting of three 15-minute extraction cycles using water as the solvent. The pH was adjusted to 8.5 or 10 with 1N NaOH at a constant temperature of 60 °C. Different flour: water ratios of 1:10 or 1:20 w/v (weight/volume) were used in each extraction cycle, coded as E1, E2, E3, and E4, and detailed in Figure 2, to evaluate the possibility of reducing the water consumption of the protein recovery process.

At the end of each extraction cycle, the remaining solid was separated from the protein solution, and fresh water at 60 °C was added at the beginning of the second and third cycles to adjust the solid-liquid ratio to the required value. The three protein solutions obtained from each extraction cycle were combined into a liquid pool and transferred to a beaker for subsequent precipitation.

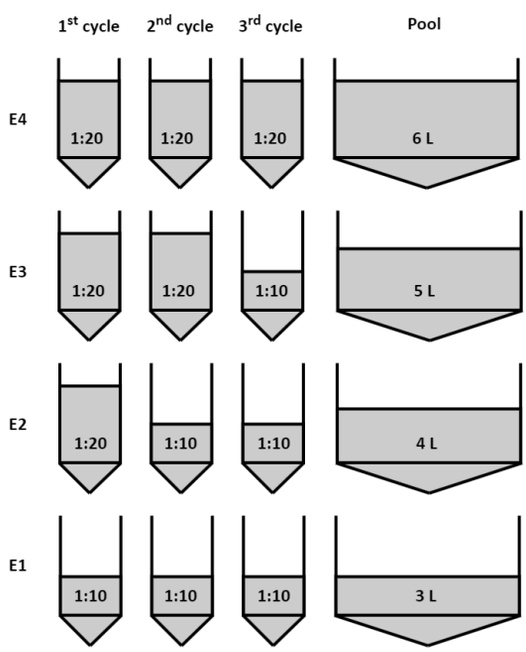

Fig. 2. Representation of the flour:water ratio (w/v) used in each extraction cycle at the alkaline extraction stage.

Isoelectric precipitation

The isoelectric precipitation stage was performed using three alternative precipitants: analytical grade 0.1 N hydrochloric acid, food grade 85% lactic acid, and a combination of GRAS freeze-dried lactic acid bacteria starters, *Lactobacillus plantarum* and *Lactobacillus lactis*, and food grade 85% lactic acid.

For the first alternative, coded as HCl, analytical grade 1 N hydrochloric acid was used to acidify the liquid pool at a temperature of 20 °C until the pH reached a value of 4.5. For the second alternative, coded as LA, food grade

85% lactic acid was used to acidify the liquid pool at a temperature of 20 °C until the pH reached a value of 4.5. In both cases, the resulting mixture was allowed to settle in a refrigerator until it reached a temperature of 4 °C, which facilitated the decantation of the protein product.

As the third alternative, coded as LAB, a partial acidification was performed with two GRAS lactic acid bacteria, *Lactobacillus plantarum* and *Lactobacillus lactis*. These bacteria were added in a proportion of 0.003 g l^{-1} in the liquid pool at a temperature of 35–37 °C for 10 hours in a thermostatic bath (Tecno Dalvo, Argentina). Once the pH reached a value of 6.5, the liquid was cooled to 20 °C and food grade 85% lactic acid was added until the pH reached a value of 4.5. The resulting mixture was allowed to settle in a refrigerator until it reached a temperature of 4 °C, which facilitated the decantation of the protein product.

Decantation, neutralization and spray drying

The liquid supernatant of the mixture obtained at the isoelectric precipitation stage was separated by decantation. Then, the pH of the precipitated suspension was adjusted to a value of 7, by adding 5N NaOH at ambient temperature with agitation during 1 hour.

Spray drying was performed in a laboratory spray dryer (TP-S15, XI'An Toption Instrument Co., Ltd, China) with co-current flow and nozzle diameter of 0.5 mm. The suspension was fed into the main chamber with a peristaltic pump at 15% of the maximum flow (2 l h^{-1}). The dry air temperature was set at 180 °C, and the resulting outlet air temperature was measured at an average value of 54–60 °C. After spray drying, the powder was collected from the cyclone and the cylindrical parts of the dryer chamber and stored in sterilized flasks for further analysis.

Performance of the protein recovery process

The performance evaluation of the protein recovery process involved the determination of the nitrogen content, recovery yield, productivity and specific water consumption. Functional properties, and antioxidant activity of the protein products were also assessed to provide a comprehensive understanding of the protein recovery process.

The nitrogen content of the protein products was assessed through AOAC method 2001.11 (AOAC 2005), where the total protein content was derived by multiplying the total nitrogen by 6.25 and reported as % db (i.e. on a dry basis or moisture-free basis). The moisture content of the protein products was determined using AOAC method 925.10 (AOAC 2005) and reported as % wb (i.e. on a wet basis).

The protein recovery yield (% db) was computed as the quantity of protein in the product obtained per kilogram of protein in the flour, as defined in Equation1.

$$Y_T = \frac{mass\ of\ protein\ in\ the\ protein\ product\ (kg\ db)}{mass\ of\ protein\ in\ the\ pea\ flour\ (kg\ db)} \quad (1)$$

The productivity of each protein recovery process (kg product db/kg flour db) was computed as the quantity of product obtained per kilogram of flour, as defined in Equation 2.

$$P_T = \frac{mass\ of\ proteinproduct\ (kg\ product\ db)}{mass\ of\ pea\ flour\ (kg\ flour\ db)} \quad (2)$$

The specific water consumption (kg water/kg final product db) was computed as the water consumed in the protein recovery process for obtaining each kilogram of the final protein product, as defined in Equation 3.

$$G_W = \frac{mass\ of\ water\ consumed\ in\ the\ protein\ recovery\ process\ (kg\ water)}{mass\ of\ proteinproduct\ (kg\ product\ db)} \quad (3)$$

Functional and antioxidant properties

Water holding capacity and oil holding capacity

Water holding capacity and oil holding capacity of the yellow peas protein products were determined according to Boye et al. (2010b), García-Vaquero et al. (2017), and Stone et al. (2015) with slight modifications. A protein product sample (0.5 g) was mixed in a 15 ml centrifuge tubes with distilled water (3 ml) or high oleic sunflower oil (3 ml) in a vortex mixer (Precytec, Argentina). The pH in each tube was adjusted to 2, 4.5, 7, 9.5, or 12, with 1N HCl or 1N NaOH, while mixed in a vortex mixer for 30 seconds. The pH-adjusted samples was centrifuged at 2200×g for 30 min in a laboratory centrifuge (Rolco, Argentina). The supernatant was decanted and the centrifuge tube containing the remaining sediment was weighed. The water holding capacity (%) and oil holding capacity (%) were computed as the quantity of water or sunflower oil retained per gram of protein product, as defined in Equations 4 and 5.

$$WHC = \frac{mass\ of\ water\ retained\ (g)}{mass\ of\ protein\ product\ (g)} \tag{4}$$

$$OHC = \frac{mass\ of\ sunflower\ oil\ retained\ (g)}{mass\ of\ protein\ product\ (g)} \tag{5}$$

Emulsifying capacity and emulsion stability

Emulsifying capacity and emulsion stability of the yellow peas protein products were determined according to García-Vaquero et al. (2017) with slight modifications. A protein product sample (containing 1 g of protein db according to Kjeldahl analysis) was mixed in a 15 ml laboratory tubes with distilled water (10 ml) in a vortex mixer (Precytec, Argentina). The pH was adjusted to 2, 4.5, 7, 9.5, or 12, with 1N HCl or 1N NaOH, while mixed in the vortex mixer for 30 seconds. To create an emulsion, high oleic sunflower oil (7.5 ml) was added to the centrifuge tube and the mixture was homogenized for 30 s at 14 000 rpm in a laboratory homogenizer (Labortechnik, Germany). An equal volume of high oleic sunflower oil (7.5 ml) was added to the centrifuge tube and the mixture was again homogenized for 90 s at 14 000 rpm in the laboratory homogenizer. The homogenized samples was centrifuged at 1100×g for 5 min in a laboratory centrifuge (Rolco, Argentina). The volume of the emulsion layer was measured. The emulsifying capacity (%) was computed as the volume of the emulsion layer relative to the total volume, as defined in Equation 6.

$$OHC = \frac{mass\ of\ sunflower\ oil\ retained\ (g)}{mass\ of\ protein\ product\ (g)} \tag{6}$$

Afterwards, the previously prepared emulsion was heated at 85 °C for 15 min in a thermostatic bath (Tecno Dalvo, Argentina), cooled at room temperature for 10 min and centrifuged at 1100×g for 5 min in the laboratory centrifuge. The emulsion stability (%) was computed as the volume of the emulsion layer after heating relative to the original volume of the emulsion layer, as defined in Equation 7.

$$ES = \frac{volume\ of\ the\ emulsion\ layer\ after\ heating\ (ml)}{volume\ of\ the\ emulsion\ layer\ (ml)} \tag{7}$$

Foaming capacity and foam stability

Foaming capacity and foam stability of the yellow peas protein products were determined according to García-Vaquero et al. (2017) and Stone et al. (2015) with slight modifications. A protein product sample (containing 0.75 g of protein db according to Kjeldahl analysis) was mixed in a 15 ml laboratory tube with distilled water (10 ml) in a vortex mixer (Precytec, Argentina). The pH was adjusted to 2, 4.5, 7, 9.5, or 12, with 1N HCl or 1N NaOH, while mixed in the vortex mixer for 30 seconds. The mixture was homogenized for 60 s at 9000 rpm in a laboratory homogenizer (Labortechnik, Germany). The volume of the foam layer was measured. The foaming capacity (%) was computed as the volume of the foam layer relative to the total volume, as defined in Equation 8.

$$FC = \frac{volume\ of\ the\ foam\ layer\ (ml)}{total\ volumen\ (ml)} \quad (8)$$

Afterwards, the previously prepared mixture was left undisturbed at room temperature, and the remaining volume of the foam layer was measured at given times (15, 30, 60, 90 and 120 min). The foam stability (%) was computed as the volume of the emulsion layer after a given time relative to the original volume of the foam layer, as defined in Equation 9.

$$FS = \frac{volume\ of\ the\ foam\ layer\ after\ heating\ (ml)}{volume\ of\ the\ foam\ layer\ (ml)} \quad (9)$$

Solubility

Solubility of the yellow peas protein products was determined according to García-Vaquero et al. (2017), and Stone et al. (2015) with slight modifications. A protein product sample (containing 0.1 g of protein db according to Kjeldahl analysis) was mixed in 50 ml centrifuge tubes with distilled water (10 ml) in a vortex mixer (Precytec, Argentina). The pH was adjusted to 7, with 1N HCl or 1N NaOH, while mixed in the vortex mixer for 30 seconds. The sample was centrifuged at 4000×g for 30 min in a laboratory centrifuge (Rolco, Argentina). A 1 g sample of the supernatant was separated, and its nitrogen content was assessed through AOAC method 2001.11 (AOAC 2005), where the total protein content was derived by multiplying the total nitrogen by 6.25. The solubility (%) was computed as the protein content after centrifugation of the supernatant of the pH-adjusted solution relative to the protein content of the full dispersion, as defined in Equation 10.

$$S = \frac{protein\ content\ \text{after centrifugation of the supernatant of}\ the\ pH\ adjusted\ solution\ (\%\ db)}{protein\ content\ in\ the\ full\ dispersion\ (\%\ db)} \quad (10)$$

Protein profiles by SDS-PAGE

Protein fractions of pea protein products were analysed by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis. A suspension of the protein powder (containing 1 mg of protein db according to Kjeldahl analysis) was solubilized in 0.125 M Tris–HCl buffer and dyed with Coommasie blue R-250. The homogenate was incubated at 85 °C for 15 minutes, followed by centrifugation at 8000g for 5 minutes at room temperature. Then, a 20 μg sample was loaded into a 12% polyacrylamide gel slab. The electrophoretic pattern of proteins was determined using a constant current of 20 mA per gel. All Blue Marker (Biorad, US) and Protein Marker II (Serva, Germany) were used as molecular weight protein markers.

Antioxidant activity

The antioxidant activity of the yellow peas protein products was determined as the free radical scavenging activity measured using the 2,2-diphenyl-1-picrylhydrazil (DPPH) method described by Calderon-Chiu et al. (2021), and Balzarini et al. (2018). A dispersion with a concentration of 0.0333 g ml^{-1} was prepared by weighting the adequate amount of the protein product and adding it to 10 ml of a 50% ethyl alcohol - distilled water solution. The dispersion was mixed for 90 minutes in a magnetic stirrer (BioSmartest, Argentina) at room temperature. Then, the remaining solid phase was filtered out. An aliquot of 2 ml of the hydroalcoholic extract was added to 10 ml of ethanolic solution of DPPH radical (0.1 mM). The mixture was stirred for 30 seconds in a vortex mixer (Precytec, Argentina), and then left to rest at room temperature for 30 min in the dark. The absorbance was measured at a wavelength of 517 nm in a UV-vis spectrophotometer (Agilent Varian Cary 50, USA). A control sample was prepared following the same procedure but without adding the protein product extract. The free radical scavenging activity (%) was computed as the absorbance of the hydroalcoholic extract of the protein product relative to the absorbance of the control sample, as defined in Equation 11.

$$DPPH = \frac{absorbance\ of\ the\ hydroalcoholic\ extract\ of\ the\ protein\ product\ (-)}{absorbance\ of\ the\ control\ sample\ (-)} \quad (11)$$

Additionally, the Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalent antioxidant capacity was determined using the method described by Luisetti et al. (2020) with slight modifications. First, a standard solution was prepared by weighting 3.2 mg of Trolox and adding it to 100 ml of 96% ethyl alcohol. Solutions with concentrations of 0, 4, 8, 16, and 32 mg l^{-1} were prepared by adding the adequate amount 96% ethyl alcohol to

the standard solution in five laboratory tubes. An aliquot of 10 ml of ethanolic solution of DPPH radical (0.1 mM) was added to each tube. The mixture was stirred for 30 seconds in a vortex mixer (Precytec, Argentina), and then left to rest at room temperature for 50 min in the dark. Then, a Trolox calibration curve was generated by measuring the absorbance of these solutions at 517 nm in a UV-vis spectrophotometer (Agilent Varian Cary 50, USA). The Trolox equivalent antioxidant activity (mg Trolox/l solution) was computed as a function of the sample absorbance at 517 nm through the Trolox linear calibration curve (R2 = 0.9905), as defined in Equation 12.

$$TEAC = \frac{0.9502 - absorbance\ of\ the\ hydroalcoholic\ extract\ of\ the\ protein\ product\ (-)}{0.0141} \quad (12)$$

Statistical analysis

Statistical analyses were conducted using one-way and/or two-way ANOVA, to ensure robust conclusions. Results were presented as mean values with standard deviations and revealed significant differences ($p < 0.05$) among experimental values, as indicated by post-hoc Tukey tests. Statistical analyses were done in R-4.3.2 software.

Results and discussion

Evaluation of the performance of the protein recovery process from yellow peas

The yellow peas used as raw material were characterized by determining their protein content, protein solubility in KOH and moisture content, which were found to be 21.92 ± 4.05% db, 90.44 ± 4.11%, and 6.03 ± 0.91% wb. These values will be used as reference to evaluate the performance of the process for obtaining protein products from such yellow peas.

Table 1 shows the performance of the protein recovery process from yellow peas for experimental runs using pH values of 8.5 or 10 in the alkaline extraction stage, and using hydrochloric acid, lactic acid (food grade), or a combination of lactic acid bacteria and lactic acid as precipitating agent in the isoelectric precipitation stage. Similar protein recovery yields ($p > 0.05$) were found for experimental runs with both pH values and every precipitating agent.

Table 1. Performance of the protein recovery process from yellow peas for different pH values in the alkaline extraction stage

	Protein content (% db)			Protein recovery yield, Y_T (% db)			Productivity, P_T (kg product db/kg flour db)			Specific water consumption, G_W (kg water/kg final product db)		
	HCl	LA	LAB	HCl	LA	LAB	HCl	LA	LAB	HCl	LA	LAB
E1 - pH 8.5	74.88 ± 2.90 a	65.68 ± 5.19 ab	49.25 ± 7.52 bc	66.76 ± 7.80 a	66.19 ± 5.77 a	66.93 ± 0.63 a	0.23 ± 0.03 c	0.26 ± 0.04 c	0.36 ± 0.05 bc	146.1 ± 22.7 a	129.0 ± 21.4 ab	94.9 ± 13.6 abc
E1- pH 10	37.64 ± 3.7 c	36.43 ± 3.51 c	32.58 ± 0.0 c	68.10 ± 1.93 a	71.96 ± 2.48 a	66.25 ± 3.66 a	0.48 ± 0.06 ab	0.52 ± 0.03 ab	0.53 ± 0.02 a	71.5 ± 9.06 bc	65.3 ± 4.04 c	63.6 ± 3.54 c

Two-way ANOVA for each variable, where different letters represent significant differences between experimental results.

A higher pH value in the alkaline extraction stage allowed obtaining larger amounts of final product per kilogram of yellow pea flour ($p < 0.05$), which also implied the usage of a smaller amount of fresh water per kilogram of obtained final product ($p < 0.05$). On the other hand, the protein content of the final product was significantly lower ($p < 0.05$) when the pH value in the alkaline extraction stage was higher. Therefore, a pH value of 8.5 was selected to be used in the rest of the experimental runs, to prioritize obtaining a protein product with a higher protein content, as it would have a better market value, by means of a recovery process with a higher expected protein recovery yield. Tanger et al. (2020) also reported protein concentrations above 70% for the alkali extractions of proteins from pea flour at pH values of 9.5 and 7, but with lower extraction yields of 46–50 %. Gao et al. (2020) observed no notable distinction in the protein content among protein products processed at pH levels of 8.5, 9 and 9.5, but the protein recovery yield exhibited a notable increase of 8 % from pH 8.5 to 9.5. For three pea cultivars, Stone et al. (2015) observed no significant differences in the protein content among products processed at pH levels of 8.5, 9, and 9.5, with a protein content of 83.3–86.9%. However, the protein recovery yield showed a significant increase of 8% from pH 8.5 to 9.5. The disparity in protein content observed with respect

to this study compared could be attributed to the preprocessing steps conducted by Stone et al. (2015), which involved dehulling and defatting prior to alkaline extraction, which entailed three cycles of hexane treatment at a solid-to-liquid ratio of 1:3 (w/v). Cui et al. (2020) found no effect of alkaline extraction pH values from 8.5 to 10 on the final protein content of protein products from four different yellow pea cultivars.

Table 2 shows the protein content, protein recovery yield, productivity and specific water consumption for the different alternatives in the protein recovery process from yellow peas, including four different combinations of solid-liquid ratios in each of the 3 cycles of the alkaline extraction stage, and three precipitant agents in the isoelectric precipitation stage.

Table 2. Performance of the protein recovery process from yellow peas

	Protein content (% db)			Protein recovery yield, Y_T (% db)			Productivity, P_T (kg product db/kg flour db)			Specific water consumption, G_W (kg water/kg final product db)		
	HCl	LA	LAB	HCl	LA	LAB	HCl	LA	LAB	HCl	LA	LAB
E1	74.88 ± 2.90 a	65.68 ± 5.19 a	49.25 ± 7.52 a	66.76 ± 7.80 a	66.19 ± 5.77 a	66.93 ± 0.63 a	0.23 ± 0.03 b	0.26 ± 0.04 ab	0.36 ± 0.05 a	146.1 ± 22.7 cde	129.0 ± 21.4 de	94.9 ± 13.6 e
E2	74.86 ± 5.40 a	69.32 ± 7.45 a	69.13 ± 3.82 a	70.37 ± 7.35 a	69.18 ± 0.78 a	68.07 ± 7.48 a	0.24 ± 0.01 ab	0.26 ± 0.03 ab	0.25 ± 0.01 ab	183.4 ± 5.9 bcd	172.6 ± 20.5 bcde	175.4 ± 9.6 bcde
E3	75.93 ± 0.51 a	70.11 ± 6.91 a	69.67 ± 8.45 a	69.4 ± 4.14 a	63.01 ± 9.62 a	64.78 ± 9.52 a	0.24 ± 0.01 ab	0.23 ± 0.01 b	0.24 ± 0.00 ab	234.8 ± 18.6 ab	240.4 ± 13.1 ab	231.8 ± 6.0 abc
E4	70.18 ± 6.59 a	69.29 ± 9.41 a	58.95 ± 0.78 a	65.81 ± 0.19 a	64.59 ± 1.20 a	59.38 ± 3.04 a	0.24 ± 0.02 ab	0.24 ± 0.03 ab	0.26 ± 0.03 ab	275.4 ± 26.7 a	277.4 ± 42.8 a	255.4 ± 33.8 ab

Two-way ANOVA for each variable, where different letters represent significant differences between experimental results.

No significant differences ($p > 0.05$) were found for the protein content of the final product and yield of the protein recovery process when different flour-water ratios were employed or when different precipitants were used. The implemented extraction strategy with the addition of fresh water in each cycle intends to simulate a continuous counter-current extraction effect, which implies improvements of the protein recovery yield with respect to the standard process with 1 or 2 extraction cycles (Accoroni et al. 2020), as the addition of fresh solvent enhances the driving force to further extract soluble proteins that are still tightly bound (Sunley 1995). A larger process productivity was observed when the extraction process consisted of three 1:10 (w/v) cycles and lactic acid bacteria were used as precipitant agent, although in general no other significant differences ($p > 0.05$) were found for the process productivity with respect to the flour-water ratios or precipitant agents.

As outlined in Rezvani et al. (2017) and Passos et al. (1994), lactic acid bacteria are known to produce lactic acid as the final product of fermentation, contributing to the acidification necessary for the pH-shifting process. However, it is noted that lactic acid production can vary significantly from theoretical predictions due to factors such as strain variability, environmental conditions (pH, temperature, and oxygen levels), and nutrients availability (including the composition of carbon and nitrogen sources in the media). In this study, the acidification of the 1000 ml liquid pool in the isoelectric precipitation stage required 6–18 ml of 1 N HCl when using this precipitant agent, while only 0.8–1.9 ml of food grade 85% lactic acid were necessary under similar operating conditions. On the other hand, when first lowering the pH to a value of 6.5 with the lactic acid bacteria, only 0.3–0.9 ml of food grade 85 % lactic acid were additionally necessary for reaching the isoelectric pH of 4.5, which represented 40–50% of the aforementioned lactic acid consumption. It is also noted that the initial mass of lactic acid bacteria added for lowering the pH was 3 mg, while 25–27 g of protein product were obtained in the different experimental runs per litre of liquid pool. In addition, based on growth kinetics models adjusted with experimental data previously proposed in the literature (Passos et al. 1994, Rezvani et al. 2017), it could be estimated that the bacterial mass could double by the end of the 10 h fermentation period. Therefore, the bacterial biomass in the final precipitate represents only a very small fraction and should not significantly by itself impact the characteristics and properties of the protein products. It is also noted that Emkani et al. (2021) previously showed that lactic acid bacteria could be used to lower the pH to a value of 4.8 during the isoelectric precipitation of pea proteins when the pH of alkaline extraction had been set at a value of 7.5, whereas such process took 6 to 10 h; while in this study, it

was found that a similar decrease in the pH value could be achieved in an equivalent timeframe, although starting from a higher initial pH value.

On the other hand, significant differences ($p < 0.05$) were found for the specific water consumption of the protein recovery process. As a general trend, the process alternatives with three 1:10 (w/v) extraction cycles and lactic acid bacteria as precipitant agent used the lowest amount of water per kilogram of obtained protein product. The performance of the protein recovery process from different matrices was previously discussed in the literature. For one extraction cycle, Rosenthal et al. (1998) found that the soy protein extraction yield decreased with increasing solid-liquid ratios. However, Sari et al. (2015) and Sunley (1995) reported higher protein yields for higher solid-liquid ratios, reaching a maximum value for ratios higher than 1:40 (with no significant differences). Shen et al. (2008) also reported higher recovery yields for tea proteins using enzymatic extraction and solid:liquid ratios above 1:35. Results here obtained for the recovery of proteins from yellow peas show that larger volumes of water are not required for achieving a better protein recovery performance.

In this context, precipitants such as lactic acid and lactic acid bacteria may be suitable substitutes for chlorhydric acid, as these precipitant agents are considered to be more environmentally friendly, are derived from natural sources and do not produce hazardous by-products (Alhamad et al. 2020). Webb and Manan and (2017) suggested that in-depth laboratory investigations have shed light on the chemical and microbiological alterations occurring during fermentation. While certain small-scale traditional fermentation processes have evolved into large-scale industries, there remains value in examining the factors conducive to such scaled-up production. Effective management of fermentation parameters include variables such as temperature, moisture levels, pH monitoring, and aeration (Garrido-Galand et al. 2021). However, due to infrastructural limitations and the prevalence of outdated technologies, Holzapfel (2002) highlights that rural areas in many developing nations have struggled to keep pace with global advancements in industrialization. Based on the obtained experimental results for productivity and water consumption, the methodology here proposed could be more easily implemented at medium size scale for obtaining a food grade protein product, which may become a relevant value adding strategy and development strategy for the Argentinian agro-industrial sector. Nevertheless, the implementation of these alternatives at larger scales would require the respective feasibility and cost evaluation.

Evaluation of functional and antioxidant properties of yellow peas protein products

The benefits of adding pea proteins to food products like emulsions, foams, or gels could potentially be enhanced by gaining a deeper understanding of how pH affects their properties (Benelhadj et al. 2016). As target food products will have different pH values, the evaluation of the impact of pH on the functional properties of the obtained protein products becomes mandatory.

For evaluating the functional properties of the protein products obtained from yellow peas, three 15-minute extraction cycles using water as the solvent with a solid:liquid ratio of 1:10 (w/v) and a pH value of 8.5 were adopted in the alkaline extraction stage (previously coded as E1). The isoelectric precipitation stage was performed using hydrochloric acid (HCl), lactic acid (LA), or a combination of lactic acid bacteria starters and lactic acid (LAB). The other parameters of the protein recovery process were kept at the values previously stated in the Methods section. Table 3 shows the experimental values for the functional properties of the protein products obtained from yellow peas. Here, the water holding capacity, oil holding capacity, emulsifying capacity, emulsion stability, foaming capacity and foam stability were determined at five pH values: 2, 4.5, 7, 9.5, and 12, while the solubility was evaluated at a pH value of 7.

Water holding capacity (WHC) and oil holding capacity (OHC)

The water holding capacity showed significant differences ($p < 0.05$) with respect to some combinations of the precipitant agent and tested pH value. However, no significant differences ($p > 0.05$) were observed in the oil retention capacity of the protein products. WHC is an important parameter to formulate food products, such as meat ones, where proteins significantly contribute to their texture. In addition, OHC of plant based proteins becomes relevant as meat substitutes to bind fat and preserve flavors (Kristo and Corredig 2015, Benelhadj et al. 2016).

For protein isolates produced from pea cultivars representing three market classes, Stone et al. (2015) found WHC values of 2.4–2.6 g g^{-1} and OHC values of 3.5–3.8 g g^{-1}, while Boye et al. (2010b) reported WHC values for protein concentrates of yellow peas, green lentils, red lentils, Desi chickpeas, and Kabuli chickpeas of 0.6–2.7 g g^{-1}, and OHC values up to about 130%, which are similar to the ones here obtained. Adebowale et al. (2011) suggested that isoelectric precipitation may alter the proteins' structure, surface area, size of macromolecule, charge, and

hydrophobicity, thus modifying or limiting their ability to interact with and absorb water and oil. This trend is also here observed, as the lowest water holding capacities were obtained for protein products precipitated with lactic acid bacteria.

Table 3. Functional properties of protein products obtained from yellow peas

pH	Water holding capacity, *WHC* (g/g)			Oil holding capacity, *OHC* (g/g)			Emulsifying capacity, *EC* (%)			Emulsion stability, *ES* (%)		
	HCl	LA	LAB	HCl	LA	LAB	HCl	LA	LAB	HCl	LA	LAB
2	3.80 ± 0.04 a	3.34 ± 0.47 ab	1.65 ± 0.08 bc	1.55 ± 0.52 a	1.40 ± 0.41 a	1.49 ± 0.19 a	65.97 ± 6.26 a	43.38 ± 9.37 ab	63.60 ± 3.39 ab	98.43 ± 2.20 a	96.70 ± 4.65 a	95.00 ± 1.51 a
4.5	1.32 ± 0.02 c	1.60 ± 0.17 bc	1.83 ± 0.22 bc	1.16 ± 0.08 a	0.83 ± 0.18 a	1.54 ± 0.40 a	ND	ND	76.66 ± 9.42 a	ND	ND	89.97 ± 14.19 a
7	2.05 ± 0.69 abc	1.25 ± 0.27 c	1.52 ± 0.00 c	1.83 ± 0.73 a	1.37 ± 0.33 a	1.85 ± 0.51 a	70.20 ± 0.28 a	66.76 ± 11.92 a	75.11 ± 1.94 a	79.68 ± 4.32 a	63.66 ± 0.03 a	86.08 ± 15.75 a
9.5	1.86 ± 0.78 bc	1.67 ± 0.86 bc	1.68 ± 0.28 bc	1.25 ± 0.38 a	1.46 ± 0.02 a	1.67 ± 0.47 a	70.33 ± 0.47 a	79.13 ± 17.63 a	73.16 ±12.13 a	94.37 ± 7.96 a	98.44 ± 2.21 a	100.00 ± 0.00 a
12	1.40 ± 0.77 c	1.19 ± 0.12 c	1.18 ± 0.04 c	0.67 ± 0.23 a	1.37 ± 0.04 a	1.38 ± 0.23 a	45.27 ± 5.10 a	76.26 ± 11.43 a	66.62 ± 7.60 a	83.40 ± 2.24 a	95.46 ± 6.41 a	86.70 ± 18.81 a

pH	Foaming capacity, *FC* (%)			Foam stability at 30 min, *FS* (%)			Foam stability at 60 min, *FS* (%)			Solubility, *S* (%)		
	HCl	LA	LAB	HCl	LA	LAB	HCl	LA	LAB	HCl	LA	LAB
2	185.29 ± 20.80 ab	135.45 ±8.09 bcde	168.58 ± 9.19 abcd	11.05 ± 4.16 ab	11.38 ± 1.57 ab	4.66 ± 0.47 bc	10.53 ± 4.19 ab	10.84 ± 2.34 ab	3.02 ± 1.45 bc	-	-	-
4.5	123.45 ± 16.53 cde	137.68 ± 31.15 bcde	131.76 ± 25.79 bcde	5.03 ± 3.58 abc	13.92 ± 3.64 a	3.58 ± 2.00 bc	4.01 ± 4.26 abc	10.67 ± 0.95 ab	2.54 ± 2.06 bc	-	-	-
7	203.24 ± 37.85 a	163.43 ± 1.08 abcd	109.94 ± 2.59 de	9.51 ± 3.51 ab	14.48 ± 2.14 a	0.27 ± 0.38 c	8.24 ± 2.48 abc	13.46 ± 1.46 a	ND	44.77 ± 0.70 b	71.30 ± 1.00 a	13.74 ± 1.56 c
9.5	181.89 ± 2.68 abc	178.68 ± 5.20 abc	137.92 ± 3.71 bcde	13.02 ± 1.45 ab	14.5 ± 1.4 a	3.85 ± 1.62 abc	12.5 ± 1.42 ab	11.71 ± 1.01 ab	0.50 ± 0.70 c	-	-	-
12	87.36 ± 1.24 e	143.32 ± 11.35 abcde	112.16 ± 17.20 de	6.99 ± 0.71 ab	8.71 ± 5.25 abc	7.32 ± 1.87 ab	5.97 ± 0.03 abc	7.41 ± 4.81 abc	5.24 ± 1.75 abc	-	-	-

Two-way ANOVA for each variable, where different letters represent significant differences between experimental results. ND = no detected activity

Emulsifying capacity (EC) and emulsion stability (ES)

The emulsifying capacity and emulsion stability of the protein products showed significant differences ($p < 0.05$) with respect to some combinations of the precipitant agent and tested pH value, as shown in Table 3. The emulsifying capacity indicates a sample's ability to swiftly adhere to the oil/water interfaces during emulsion formation, preventing flocculation and coalescence. This property is relevant to the elaboration of many food products by improving texture, preventing oil/water phases separation, and retaining flavors. Stable emulsions aid in distributing flavors, thus making food products more palatable (Naurzbayeva et al. 2023).

It is noted that no emulsion was formed at a pH value of 4.5 when using hydrochloric acid or lactic acid as the precipitant agent during the protein recovery process, since the solubilized protein product rapidly coalesced and precipitated when the pH was adjusted to this value, which corresponds to the isoelectric point of the proteins. Similarly, Ladjal-Ettoumi et al. (2015) observed a minimum emulsifying capacity for pea protein isolates at a pH value of 4.5.

The emulsifying capacity has been shown to be correlated with the solubility index, being higher for pH values of 2, 7, or 9.5, where the solubility index is usually higher (Shand et al. 2007, Adebiyi and Aluko 2011, Ladjal-Ettoumi 2015). On the other hand, the LAB-precipitated protein products exhibited emulsifying capacity even at the isoelectric pH value. According to Aluko et al. (2009), pea protein products obtained by means of lactic acid

bacteria precipitation presented higher sugars contents, which may potentially enhance the solubility of the obtained products, thus improving their emulsifying capacity. Stone et al. (2015) determined the emulsifying properties of pea protein isolates prepared using alkaline extraction - isoelectric precipitation, or salt extraction - dialysis, and found, for the former method of preparation, values of the emulsion capacity and emulsion stability at pH 7 of 187.5–193.7 g g^{-1} and 96.7–99.9 %, respectively. From the previous literature, they also retrieve contradictory accounts regarding the influence of the production method of legumes protein products on their emulsion capacity, where some authors found an improvement of this property when using salt extraction, ultrafiltration, micellar precipitation or pH-shifting, while other works didn't report such differences (Boye et al. 2010a, Adebowale et al. 2011, Karaca et al. 2011). According to Stone et al. (2015), high values of the emulsion stability imply that the added oil is thoroughly dispersed in the aqueous phase due to adequate strength of the viscoelastic film at the interface, as well as sufficient charge repulsion and/or steric hindrance between droplets to prevent their coalescence.

Foaming capacity (FC) and foam stability (FS)

As shown in Table 3, the foaming capacity of the protein products showed significant differences ($p < 0.05$) with respect to some combinations of the precipitant agent and tested pH value. The foam layer height was measured every 15 minutes after the foam was formed, whereas the foam stabilities at 30 and 60 minutes are reported in Table 3. The ability of pea flours to form foams could be deemed essential for their application in the production of non-dairy foods, as foam formation is essential in the manufacturing of various food products, including ice cream, cakes, and meringues, which underscores the significance of this functional property for the utilization of pea flour within the food industry (Aluko et al. 2009). The foaming capacity indicates the ability of the recovered proteins to adsorb to the air–water interface and decrease the interfacial tension, and the foam stability relates to the strength of the interfacial film of the adsorbed proteins

As a general trend, it is observed that the foam is more stable (with a significance level of 95% for most tested pH values) when formed with protein products obtained using hydrochloric acid or lactic acid as precipitant agent during the protein recovery process, when compared with the foam formed with protein products obtained using lactic acid bacteria. Stone et al. (2015) reported foaming capacity values of 155.0–183.3 % for pea protein isolates produced by alkali extraction-isoelectric precipitation, while the foam stability value was 68.0–69.6 % after 30 minutes, noting that the values of both functional properties were dependent on the extraction and drying method.

For pea protein isolates, concentrates and flours, Aluko et al. (2009) found that foam formation was dependent on the pH value, protein content and particle size of the samples, which influenced the availability of the foaming agents and their ability to form stable air-water interfacial membranes with adequate protein-protein interactions. In addition to these variables, Chao and Aluko (2018) reported that the foaming capacity and foam stability of pea protein isolates depended on the heat pretreatment temperature due to the alteration of the protein structure, modifying the polypeptide flexibility and charges on the interfacial protein membranes (Lam et al. 2018). Based on their experimental results and previous ones reported in the literature, Pei et al. (2022) also reported that the foaming capacity and foam stability of protein products may be negatively impacted by the usage of lactic acid bacteria, since fermentation may expose excess hydrophobic groups and promote aggregation of proteins and LAB cells, decreasing the migration of proteins to the interface and reducing their foaming properties.

Protein solubility (S)

The protein solubility was significantly higher ($p < 0.05$) for protein products obtained using lactic acid as a precipitant agent during the protein recovery process, with respect to the solubility of protein products obtained using hydrochloric acid, as seen in Table 3. As many authors reported for pea protein products (Shand et al. 2007, Adebiyi and Aluko 2011, Ladjal-Ettoumi 2015), the alkaline-soluble proteins (i.e. the ones recovered through the pH-shifting process) exhibit a typical bell-shaped curve, with a minimum solubility index around the isoelectric point (4.5–5.0) and maximum values under alkaline (pH 8) and very acidic (pH 2) conditions.

Cui et al. (2020) reported a solubility index at pH 7 of 50–80 % for protein products obtained from yellow pea cultivars with similar initial protein contents, using hydrochloric acid as a precipitant agent. Even so, they concluded that the solubility tested at different pH values resulting from protein products obtained with different alkaline extraction conditions is case-dependent, as contradictory results were previously reported in the literature as a function of the specific protein conformation, protein surface charge, and ionic strength during protein extraction. Ladjal-Ettoumi et al. (2015) studied the solubility of pea, chickpea and lentil proteins, and concluded that the

solubility profiles for different pH values closely agree with their zeta potential profiles, thus being dependent upon their surface net charge. Chao and Aluko (2018) found values of about 28% for the solubility of pea protein isolates obtained using hydrochloric acid at pH 7, which weren't significantly modified by exposing the sample to a water bath with a temperature up to 90 °C, which suggested that no excessive aggregation occurred due to the heat treatment that would have reduced the protein-water interactions.

The solubility of protein products obtained using lactic acid bacteria had the lowest value ($p < 0.05$), as shown in Table 3. Emkani et al. (2022) reviewed the state of the art regarding lactic acid fermentation of legumes and legumes and found contradictory accounts of its impact on protein solubility, depending upon factors such as production of acid by certain types of microorganisms, changes in the proteins surface, occurrence of proteolysis, diminution in the protein size, degradation of starch, among others. As an example, Shi et al. (2021) evaluated implementing 5 to 30 hours of lactic acid fermentation as a strategy to improve the sensory and functional properties of pea proteins, where they concluded that fermentation changed the recovered protein configuration by exposing their hydrophobic groups, subsequently reducing their solubility in water.

Protein profiles by SDS-PAGE

Figure 3 shows the SDS-PAGE profiles of the three protein powders obtained using HCl, LA, and LAB. The pea protein profiles of all samples revealed polypeptide subunits ranging from 10 to 75 kDa, consistent with previously reported characteristics of pea proteins (Ladjal-Ettoumi et al. 2015). The polypeptide profiles of the three samples exhibited compositional similarities, particularly regarding the presence of polypeptide subunits of Legumin (basic subunit, ~25 kDa). HCl sample exhibits a higher intensity band for legumin (acidic subunit, ~37 kDa) and vicilin α, β, and γ subunits (~50 kDa). However, the convicilin subunit (~71 kDa) is only visible in the HCl sample. Consequently, it is observed that lactic fermentation and acidification resulted in a loss of the convicilin band (~71 kDa). This phenomenon was also observed by Emkani et al. (2021) in pea protein samples fermented with *Streptococcus thermophilus, Lactobacillus acidophilus, and Bifidobacterium lactis*, and may be attributed to proteolysis during legume fermentation. During pea fermentation, peptide proteolysis and hydrolysis aid in the conversion of amino acids into bioactive forms (Zhao et al. 2016), although proteases may also attach to substrates with akin chemical compositions, thereby possibly diminishing process yields. Therefore, to address this limitation, more selective processes could be designed using specific enzymes to enhance process efficiency (Siddiqui et al. 2023).

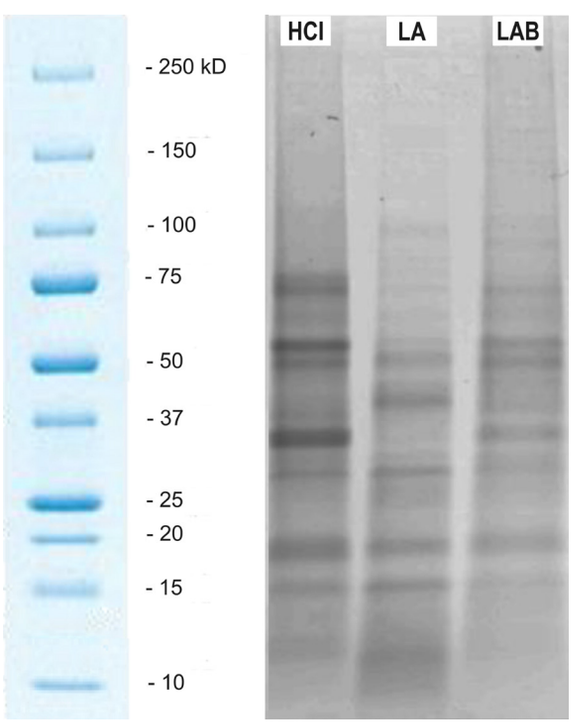

Fig. 3. Protein profiles by SDS-PAGE of three protein powders obtained using hydrochloric acid (HCl), lactic acid (LA), or combination of lactic acid bacteria and lactic acid (LAB).

Antioxidant activity

Table 4 shows the experimental values for the antioxidant activity of the protein products from yellow peas, obtained through the ph-shifting process with three 15-minute extraction cycles using water as the solvent with a solid:liquid ratio of 1:10 (w/v) and a pH value of 8.5 in the alkaline extraction stage (previously coded as E1), and using hydrochloric acid (HCl), lactic acid (LA), or a combination of lactic acid bacteria starters and lactic acid (LAB) in the isoelectric precipitation stage. Significant differences ($p < 0.05$) were found between the antioxidant activity of the protein products obtained using LAB as a precipitating agent, with respect to the protein products obtained using only HCl or LA. Emkani et al. (2022) found that fermentation with lactic acid bacteria of protein products from different pea varieties could improve the scavenging capacity of DPPH and ABTS radicals, where the antioxidant activity and the inhibition of free radicals usually increased as the fermentation progressed.

Table 4. Antioxidant properties of protein products obtained from yellow peas

	HCl	LA	LAB
Free radical scavenging activity (%) DPPH (%)	3.47 ± 0.25 b	4.16 ± 0.31 b	9.81 ± 0.69 a
Trolox equivalent antioxidant activity TEAC (mg Trolox/l solution)	0.48 ± 0.22 b	0.96 ± 0.6 b	11.21 ± 0.43 a
Trolox equivalent antioxidant activity TEAC (mg Trolox/100 g sample)	1.47 ± 0.66 b	2.91 ± 1.83 b	33.67 ± 1.32 a

One-way ANOVA for each variable, where different letters represent significant differences between experimental results.

The antioxidant activity of all protein products was lower ($p < 0.05$) than that of the yellow peas, whose values were 83.54 ± 23.61 mg TEAC/100 g sample. Similarly to the values here found, Han et al. (2008) reported a total antioxidant activity value of 0.85 mg TEAC/g for yellow peas, whereas the values for other legumes could be 4–5 times larger. For ten pea cultivars, Chen et al. (2023) reported DPPH scavenging activities of 1.51–3.12 mg TEAC/g, where the differences between varieties in the amount of measured phenolic compounds might be due to their structure and extraction method.

Conclusions

The alternative pH-shifting processes for the recovery of yellow pea proteins resulted in products with comparable protein contents and similar protein recovery yields, when different flour-water ratios were employed in the alkaline extraction or when different precipitants were used for the isoelectric precipitation. However, significant differences ($p < 0.05$) were found for the specific water consumption of the protein recovery process (94.9 kg water/kg protein product) for a flour-solvent ratio of 1:10 (w/v) and lactic acid bacteria as precipitant agent, while maintaining the productivity levels (0.36 kg protein product/kg pea flour).

Furthermore, the protein products obtained with hydrochloric acid, lactic acid, and/or lactic acid bacteria presented acceptable functional properties for a wide range of pH values, including solubility, water and oil retention, and foaming and emulsifying capacities. It was noted that the LAB precipitated protein products exhibited emulsifying capacity even at the isoelectric pH value, and presented a high antioxidant capacity of about 40% of that of the cultivar. These physicochemical and functional attributes would render yellow pea proteins as a versatile food ingredient for usage into a wide range of formulations, including bakery and dairy items, gluten-free foods, dressings, and other innovative products. In addition, the resultant pH-shifting process could be adopted by small and medium-sized companies for adding value to yellow peas through a sustainable and technically feasible protein recovery alternative.

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